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BIOREMEDIATION OF FLUAZIFOP-p-BUTYL HERBICIDE BY SOME SOIL BACTERIA ISOLATED FROM VARIOUS REGIONS OF TURKEY IN AN ARTIFICIAL AGRICULTURAL FIELD

The bioremediation rate of fluazifop-p-butyl ($C_{19}H_{20}F_3NO_4$) was monitored. Bacteria were isolated in agricultural soil samples. Fifteen sterilised glass jars were inoculated with 2, 5, 10, 20 cm^3 of a homogenised bacterial mixture (10^9 CFU/cm^3), then sterile agricultural soil and 60 μg of fluazifop-p-butyl (in liquid form) were added to each jar. Each week, filtrated water drained from bottles was analysed for fluazifop-p-butyl concentration, chemical oxygen demand (COD), biochemical oxygen demand (BOD_5) and total organic carbon (TOC). Additionally, pH and dissolved oxygen concentration were monitored. The highest biodegradation rate was observed in the soil sample containing 20 cm^3 of the culture media. In this media, fluazifop-p-butyl, COD, BOD_5 and TOC removals were measured as 91, 83, 96 and 86%, respectively, at the end of the 2 months. The DO level was measured between 3 and 6 mg O_2/dm^3 in the first month for all cultures. An increase of pH was recorded during the first month and after this time a pH decrease was noted.

1. INTRODUCTION

Today, to handle food requirements for the increasing population in the world, farmers are using high amounts of herbicides. But this situation brings some environmental health risks. The pesticide industry is also conducive to introducing a high amount of toxic herbicides into the environment [1]. The increased use of pesticides has caused both environmental and public health concerns [2]. Pesticides are widely used in agriculture to improve production, protect stored crops, and control disease vectors. Although pesticide usage has benefits, the health risks have been associated with nontarget subjects including humans who are occupationally and/or environmentally exposed to

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these agrochemicals [3]. Herbicides are the main pollutants in receiving environments. According to the latest investigations, from 2015, it was understood that 391 000 tons of active ingredients were applied to receiving environments but that includes the carbon dioxide used to protect pesticide stocks as well as non-farm uses, such as in forestry [4]. Pesticides affect the microbiological activity in the soil ecosystem by changing the population of phosphate and cellulolytic solubilizing bacteria and accordingly changing the ammonification of soil and also the nitrogen balance [5]. Some factors play a high role in bioremediation rates of the pesticides. Soil properties affect pesticide degradation and adsorption, helping in their degradation from the soil [6]. Additionally, some bacterial populations degrade hydrocarbons [7]. Alternative low-cost biological methods of remediation are more effective than other physical and chemical methods. These friendly biotechnologies have low productivity as only a few microorganism strains can perform the full bioremediation of pesticides [8]. Microorganisms are thought to play an important role in the removal and detoxification of pesticides from the environment. Many bacteria that can degrade carbamate pesticides have been isolated from soil around the world [9]. Receiving environment has been stated to be the most threatening accordingly that pesticides can affect the indigenous organism in the soil and corrode the food chain from bottom to top [10]. Bioremediation of pesticides takes a long serious time because degrading bacteria in agricultural field constitute only about 10% of the total population. The increase of bioremediation rate *in situ* may be carried out in the bioremediation applications [11]. Conventional approaches (e.g., landfilling, recycling, pyrolysis and incineration) to the remediation of contaminated sites are inefficient and costly and can also lead to the formation of toxic intermediates [12]. Thus, biological decontamination methods are preferable to conventional approaches because, in general, microorganisms degrade numerous environmental pollutants without producing toxic intermediates [13].

Bacteria in nature could degrade pesticide residues with low cost and environmentally friendly without secondary pollution [14]. But the efficiency was relatively slow, and the natural environment was complex and changeable, which may affect the feasibility and efficiency of microbial degradation of pesticides [14]. Factors that influence the rate of pesticide degradation by microorganisms are either related to the microorganisms and their biological factors or associated to the environmental factors such as rainy days, hours of sunshine, soil temperature, soil pH, water holding capacity, organic matter ingredients, etc. Immobilization increases bacterial opposition to adverse environmental factors [15].

Bioremediation is non-invasive, eco-friendly and also cheaper than physical and chemical methods, and can end with biodegradation or transformation of environmental pollutants to less toxic or white forms [16]. Bioremediation in soil environment can be executed out in a particularly treated place (*ex situ*) or at the place of contamination (*in situ*). *In situ* application is used if there is no opportunity to remove contaminated soil, for example when pollution affects a large-scaled field [17].

Fluazifop-p-butyl ($C_{19}H_{20}F_3NO_4$) is a selective phenoxy herbicide used for post-emergence control of annual and perennial grass weeds. This kind of herbicide is used for corn,

peanuts, soy sprouts, cotton, potato and sugar cane. These agricultural products are produced in Marmara, Mediterranean and East Anatolian regions of Turkey and also other regions with the Mediterranean and continental climate all over the world. Various scientists have been studying the biodegradation of fluazifop-p-butyl using certain microorganisms [18].

The main goal of the present laboratory-scale research study was to assess the bioremediation of herbicide fluazifop-p-butyl with important environmental parameters such as pH, dissolved oxygen, COD, TOC and BOD_5 with the addition of different bacteria concentrations. The recommended dosage of herbicide is 15 g/(dm^3·ha) for tomato, vineyard, canola, masoor, cotton, potato, sugar beet and onion cultivation. The research results would be useful to scientists who need to develop new alternative methods for the treatment of pesticides like fluazifop-p-butyl herbicide which are differently otherwise to conventional biological wastewater treatment.

2. MATERIALS AND METHODS

Chemicals and reagents. Fluazifop-p-butyl active ingredient was obtained from Sigma-Aldrich (Germany) with a CAS number of 79241-46-6 while Malt extract agar (MEA) from Sigma Aldrich (Turkey) with a lot number of M6409. Plate count agar (PCA) and sabouraud dextrose broth (SDB) were purchased from Merck with a catalogue number of 146249 and 146366, respectively.

Bacteria used in the study. In this study, *Microbacterium chocolatum*, *Brevibacterium macrolides*, *Bacillus macroides* (from corn farming area of Marmara Region), *Ochrobactrum thiophenivorans*, *Sphingomonas melonis*, *Sphingomonas aquatilis* (from cotton farming area of Mediterranean Region) and *Bacillus subtilis* (from tomato farming area of East Anatolian Region) bacteria species were identified and used. The bacterial strains were retained in Petri dishes on plate count agars at 4 °C in the refrigerator. These microorganisms were available in our stock culture collections. Accession numbers and accuracy identification rates of these bacteria are given in Table 1.

Table 1

Identified approximate bacterial species

Accession number	Approximate species	Identity [%]
JX448376.1	*Brevibacterium macroides*	89
AJ491708.1	*Bacillus macroides*	87
CGMCC4436	*Microbacterium chocolatum*	90
NC000964.3	*Bacillus subtilis*	90
AM490617	*Ochrobactrum thiophenivorans*	91
AVM11_16345	*Sphingomonas melonis*	90
JCM11455	*Sphingomonas aquatilis*	90

Bioremediation studies. All bacteria were cultured on plate count agar (PCA) at 27 °C slants in a glass tube for preparation of inoculum. After the incubation period of seven days, conidial suspensions were used for the preparation of inoculum. 1 cm^3 of this consortia includes approximately 1×10^9 colony-forming unit of bacteria (CFU) was transferred into a sterile 100 cm^3 flask containing 99 cm^3 of Sabouraud's dextrose broth (SDB) and agitated on a rotary shaker at 130 rpm for 7 days at 27 °C. After one week, flask contents were homogenised and used for COD and BOD_5 (1.5–2.5 g O_2/dm^3) studies under submerged culture conditions.

2, 5, 10 and 20 cm^3 of the enriched homogenised culture mix (each cm^3 contains approximately 10^9 CFU bacteria) were transferred into glass jars with a depth of 15 cm and surface area of 400 cm^2 filled with agricultural soil (sterilised at 105 °C for four days) that had not been exposed to herbicides or other PAHs previously (this situation has been confirmed with LC-MS-MS analyses). One jar was used for blank studies. The control unit (without bacteria) used as blank jar contained only fluazifop-p-butyl and sterile agricultural soil. 700 g of soil was mixed with 60 μg of fluazifop-p-butyl (in liquid form). This means 85 ng of herbicide/g of soil. The suggested dosage for tomato, vineyard, canola, masoor, cotton, potato, sugar beet and onion farmers was 15 g/dm^3/ha. The caps of all jars were staved with holes of 0.2 mm in diameter.

Every week, the soil in each unit was mixed with 250 cm^3 of sterilised tap water to obtain filtrate water. The water collected from the caps of the jars was filtered again through 0.45 μm pore size Whatman filter (Cat. No. WHA10401114) and used to determine the COD, BOD_5, TOC, fluazifop-p-butyl concentration and changes in pH and dissolved oxygen (DO) concentration for eight weeks. For each parameter, each experiment was performed in triplicate and a total of 15 portions of soils was used in the study for each experiment and average results were calculated.

For fluazifop-p-butyl determination, the EPA Method 535 (measurement of chloroacetanilide and other acetamide herbicide degradation products in drinking water by solid-phase extraction and liquid chromatography/tandem mass spectrometry) was used. The COD experiments were performed with HACH DRB 200 thermoreactor and Hach COD kits that are usable in the range of 0-1500 mg O_2/dm^3 (Cat number: 23459-52) by the line of closed reflux method (Standard Method 5220C). The fluazifop-p-butyl concentration was monitored weekly. The BOD_5 analyses were performed with AL606 Oxitop device according to the Standard Method 5210B (5-day BOD_5) and TOC determinations were performed with Standard Method 5310A (burning at a high temperature) with Tekmar-Dohrmann-Apollo 9000 device [19]. All of the experiments were performed at room temperature (25 °C).

Statistical analyses. Statistical analyses were done with Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA). The values are the averages of the results of three replicates of each experiment with a standard error (SE). To compare the decrease of fluazifop-p-butyl concentration as well as COD, TOC and BOD_5 reduction, the data were analysed by analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

As the microorganism and nutrients were added to the soil in glass jars, the increase of COD and BOD_5 was observed (as compared to the blank sample). At the same time, a significant weekly increase in the removal rates of COD and BOD_5 was stated.

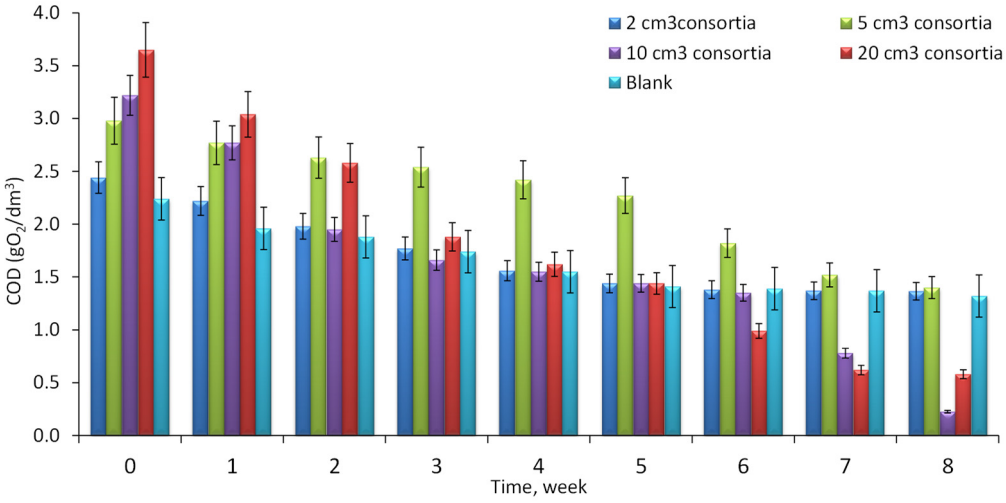

Fig. 1. Time dependences of COD for various contents
of homogenised bacterial mixture (2–20 cm^3) in the sterile agricultural soil

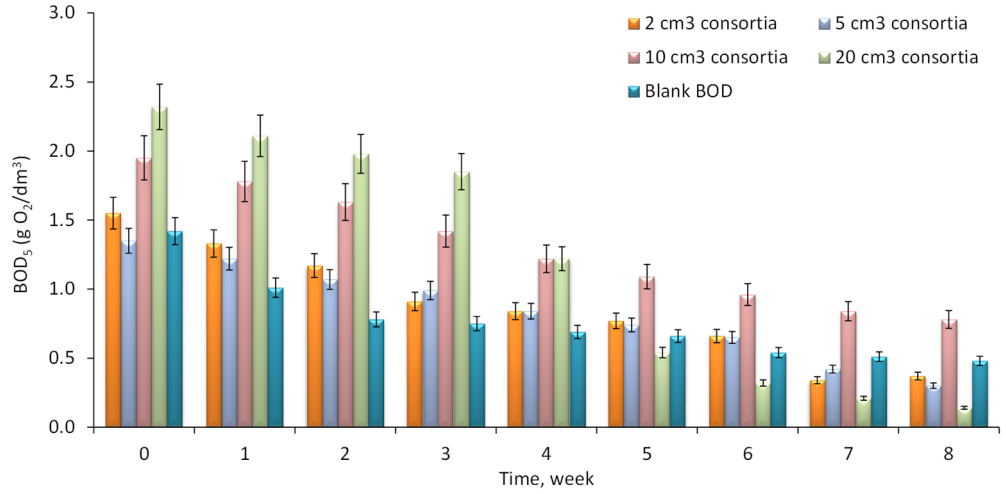

Fig. 2. Time dependences of BOD_5 for various contents
of homogenised bacterial mixture (2–20 cm^3) in the sterile agricultural soil

To be sure there is no fluazifop-p-butyl was used in the soil previously, its residuals were checked and verified. When there is no fluazifop-p-butyl in the soil there is an existing COD and BOD_5 in the filtrated water. These COD and BOD_5 are the values originating from carbon residuals in the soil. COD and BOD_5 values in the filtrate obtained from the soil were nearly the same every week (25–50 mg O_2/dm^3) and there were no differences between the removal rates, so these values were ignored.

According to the results of the COD and BOD_5 analyses, at the end of the two months, the best COD reduction amounting to 84% occurred in the 20 cm^3 mixed culture (Fig. 1), with a BOD_5 reduction of 94% (Fig. 2). This rate was similar to that of the 10 cm^3 mixed culture (93% BOD_5 removal). The reduction rate of COD on the 10 cm^3 mixed culture was 60%. COD and BOD_5 reduction rates were 38% and 66%, respectively, in a blank medium. In the 2 cm^3 mixed culture, reduction rates were 44% and 78%, respectively. The COD reduction rate was 53% in the 5 cm^3 mixed culture. Although fluazifop-p-butyl amounts used in all soil media were in equal concentrations, COD and BOD_5 results were different because of carbon residuals. In the first period of the study, the COD and BOD_5 values of samples supplemented with 2, 5, 10 and 20 cm^3 mixed cultures were increasing gradually, being higher than for blank samples. These values increased as well on the first fluazifop-p-butyl application date.

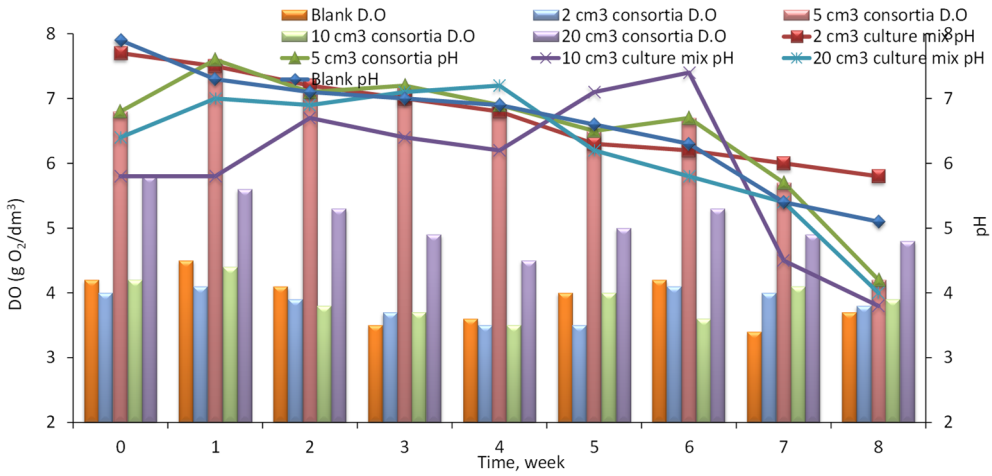

Fig. 3. Time dependences of DO and pH for various contents of homogenised bacterial mixture (2–20 cm^3) in the sterile agricultural soil

In Figure 3, the dissolved oxygen contents show a regular increase in the first 3 weeks in 2 cm^3 and 5 cm^3 mixtures and 2 weeks for blank, 10 cm^3 and 20 cm^3 mixtures. This parameter decreased after the third week and then increased again in some different culture conditions. This situation can be explained with the fact that the soil units including different concentrations of microorganisms were ventilated and microbial bio-

remediation seen in aerobic conditions. These microorganisms exert their bioremediation adeptness in aerobic conditions, so microbial communities in the soil are facultative anaerobes.

pH values decreased over time and thus, the filtrate obtained from the soil became acidic for blank, 2 cm^3 and 5 cm^3 mix media. In 10 cm^3 and 20 cm^3 media, this parameter increased in weeks 6 and 4, respectively, and then decreased again (Fig. 3). pH and temperature of most of the biosurfactants are not affected by the environmental conditions. Recent laboratory research suggested that lichenysin, which is developed by soil bacteria *B. licheniformis* was less affected by temperature (up to 50 °C) and pH between 4.5–9.0 [20]. Many environmental impacts can affect coagulation and flocculation processes such as mixing speed, pH, and dosage of pesticide active material, retention time, and temperature [21]. The effect on pH can be explained by CO_2 accumulation induced by carbon dioxide emitted to the soil media. Carbonic acid appears because of the reaction of water with carbon in the herbicide or carbon dioxide that penetrated the soil, as well as through microorganism activity [22].

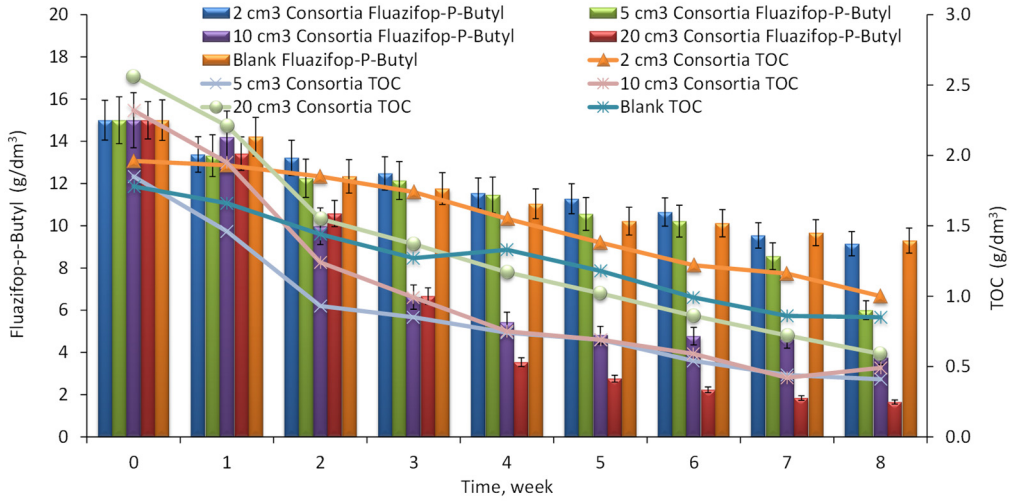

Fig. 4. Time dependences of fluazifop-p-butyl content and TOC for various contents of the homogenised bacterial mixture (2–20 cm^3) in the sterile agricultural soil

The most active fluazifop-p-butyl degradation performance equal to 89% was seen in 20 cm^3 mixed culture. In the same media, for the TOC parameter, the degradation rate was 87%. In the 10 cm^3 mixed culture, these rates were 75% and 79%, respectively, at the end of the eighth week. At the same time, in the 5 cm^3 mixed culture medium, degradation rates for these parameters were 60% and 78%, respectively. The active ingredient removal rate was 39% in the 2 cm^3 mixed culture media, with 34% in the blank

media. TOC removal rates were 80% and 49% in the 2 cm^3 and blank media, respectively, at the end of the eighth week. Since the herbicide concentration was the same in all media, different results for the TOC parameter suggest that there was a change in the culture media. As the nutrients in the blank media and the mixed culture concentrations increased gradually, the TOC values also increased. As the microorganism concentrations in the soil media increased, the TOC reduction rate also increased (Figure 4).

Biodegradation of pesticides by soil microorganisms involving seven different isolates of Pseudomonas, Agrobacterium and Bacillus has been studied [23]. *B. cereus*, *B. subtilis*, *B. melitensis*, K. species, *P. aeroginosa*, *P. fluorescens*, and *S. marcescens* are capable of degrading 46–72% of chlorpyrifos as a sole carbon source in a sedimentary medium after incubation of three weeks [24].

Mohammadi and Nasernejad [25] demonstrated that arrest of *Phanerochaete chrysosporium* on sugarcane considerably changed the activity and production of manganese peroxidase during the biodegradation of anthracene. After the immobilization of *Acinetobacter venetianus*, a higher rate of tetradecane degradation was occurred.

Increased bioremediation rate of phenol by immobilised *Candida tropicalis* on persistent organic pollutants was also demonstrated [26]. The bacterial strains are able for bioremediation in bioreactors because they remain active for up to 8 bioremediation cycles [27]. Emtiazi et al. [28] found that *Escherichia coli* immobilised on perlite were more genetically stable than in other carriers, and they could produce biosurfactants, which increased the degradation degree of petroleum hydrocarbons. Erguven and Demirci [29] monitored the bioremediation performance of *Ochrobactrum thiophenivorans* and *Sphingomonas melonis* bacteria and their consortia to reduce the imidacloprid pesticide in soil media. After 14 days period, they found full reduction rates for imidacloprid active material for each bacterium and their mixtures while COD reduction rates were 97% and 96% for two types of bacteria. Additionally, they investigated TOC and BOD_5 removal rates. 97% reduction seen for both types and their consortia. Yáñez-Ocampo et al. [30] demonstrated bioremediation of a methyl-parathion and tetrachlorvinphos mixture by a mixture of microorganism immobilised on tezontle. As a result of their research, the death of free cells occurred after one week. Bioremediation can be a sensible method for soil pollution with fluazifop-p-butyl herbicide. Bioremediation of different types of soil bacteria was positively enhanced in the soil system. According to these results, there was a suitable bacterial species in agricultural fields in different regions of Turkey for bioremediation of these types of herbicide-contaminated liquid and soil media. The fluazifop-p-butyl degrading bacterial isolates obtained as a result of this study exhibited strong fluazifop-p-butyl degradation potential and were able to conduct soil bioremediation with fluazifop-p-butyl concentration as high as 85 ng/g of soil.

In a blank medium, removal rates were observed between 38% and 66% for fluazifop-p-butyl, COD, BOD_5 and TOC parameters at the end of 8 weeks. This could be explained also with the half-life of the pesticide in soil media, especially with the adsorption mechanism. In the soil system, different concentrations of microorganisms

were tested, and the best reduction rates for active material were observed with the 20 cm^3 (approximately 20 × 109 CFU/cm^3) mixed culture, with 89% reduction at the end of the eighth week. At the end of this period, COD, TOC and BOD_5 removal yields in the 20 cm^3 mix medium were observed as 84%, 87% and 94%, respectively. Same changes observed in fluazifop-p-butyl, COD, TOC and BOD_5 values were explained with the fact that increasing concentrations translate into increased activities of microorganisms.

4. CONCLUSIONS

The consortia of *Brevibacterium macrolides*, *Bacillus macrolides*, *Microbacterium chocolatum*, *Bacillus subtilis*, *Ochrobactrum thiophenivorans*, *Sphingomonas melonis* and *Sphingomonas aquatilis* bacteria could break down the fluazifop-p-butyl in the soil medium. But this reduction down never accesses to zero for COD, TOC and BOD_5 parameter. Because there are still microorganisms and these COD, TOC and BOD_5 values are came from sabouraud dextrose broth media. Additionally, reduction in active material can give us opinion about high bioremediation efficiency.

In summary, bioremediation can be an alternative and highly effective method to reduce fluazifop-p-butyl or this kind of pesticide contaminations. Agricultural soils contain microorganisms with the ability to bioremediate persistent organic pollutants. Since pesticides are applied to crops, the soil is the medium that mostly gets these chemicals and also in live organisms. Generally, bacteria that have been identified as biodegrades have been isolated from pesticide-contaminated fields. It is understood that there were too many microorganisms to reduce the negative effects of pesticides in agricultural soils. To remediate the fields from the pesticides, it is recommended to use consortia of *Brevibacterium macrolides*, *Bacillus macrolides*, *Microbacterium chocolatum*, *Bacillus subtilis*, *Ochrobactrum thiophenivorans*, *Sphingomonas melonis* and *Sphingomonas aquatilis* consortia. The results obtained from the study demonstrate a high potential for fluazifop-p-butyl remediation using some soil microorganisms isolated from different agricultural regions of Turkey. The COD, BOD_5 and TOC parameters can be helpful for researchers about removal rates. It was also understood that these isolated bacteria used the fluazifop-p-butyl as a nutrient.

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